JOURNAL OF VIROLOGY, Feb. 1982, p. 435–448
0022-538X/82/020435-14$02.00/0

Vol. 41, No. 2

Characterization of Proviruses Cloned from Mink Cell Focus-Forming Virus-Infected Cellular DNA

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Received 26 August 1981/Accepted 6 October 1981

Two proviruses were cloned from *Eco*RI-digested DNA extracted from mink cells chronically infected with AKR mink cell focus-forming (MCF) 247 murine leukemia virus (MuLV), using a lambda phage host vector system. One cloned MuLV DNA fragment (designated MCF 1) contained sequences extending 6.8 kilobases from an *Eco*RI restriction site in the 5′ long terminal repeat (LTR) to an *Eco*RI site located in the envelope (*env*) region and was indistinguishable by restriction endonuclease mapping for 5.1 kilobases (except for the *Eco*RI site in the LTR) from the 5′ end of AKR ecotropic proviral DNA. The DNA segment extending from 5.1 to 6.8 kilobases contained several restriction sites that were not present in the AKR ecotropic provirus. A 0.5-kilobase DNA segment located at the 3′ end of MCF 1 DNA contained sequences which hybridized to a xenotropic *env*-specific DNA probe but not to labeled ecotropic *env*-specific DNA. This dual character of MCF 1 proviral DNA was also confirmed by analyzing heteroduplex molecules by electron microscopy. The second cloned proviral DNA (designated MCF 2) was a 6.9-kilobase *Eco*RI DNA fragment which contained LTR sequences at each end and a 2.0-kilobase deletion encompassing most of the *env* region. The MCF 2 proviral DNA proved to be a useful reagent for detecting LTRs electron microscopically due to the presence of nonoverlapping, terminally located LTR sequences which effected its circularization with DNAs containing homologous LTR sequences. Nucleotide sequence analysis demonstrated the presence of a 104-base-pair direct repeat in the LTR of MCF 2 DNA. In contrast, only a single copy of the reiterated component of the direct repeat was present in MCF 1 DNA.

Dual-tropic murine leukemia viruses (MuLV's) have been isolated from mouse-passaged Moloney MuLV (MoMuLV) thymic lymphomas (14, 20) and other spontaneous and induced viral leukemias (36, 43). Because of their association with mouse neoplasms and their capacity to accelerate some types of lymphomas (6, 27, 28, 36), the mink cell focus-forming (MCF) MuLV's have been suspected of playing an important role in the development of certain mouse tumors. Based on serological and biological characteristics of MCF viruses (11, 14), as well as RNase T_1 oligonucleotide fingerprinting analysis, heteroduplex mapping studies, and examination of their envelope glycoproteins (4, 10, 22, 35, 42), it has been suggested that MCF MuLV's arise after a recombination event between an ecotropic MuLV and envelope determinants related to xenotropic MuLV's.

To more fully characterize the molecular structure of the AKR MCF 247 MuLV genome, we cloned the proviral DNA present in infected mink cells, using a lambda phage vector system. Of the two clones obtained, one, measuring 6.8 kilobases (kb) in size, appeared to be colinear with the MCF 247 *Eco*RI fragment extending from the 5′ long terminal repeat (LTR) to the envelope (*env*) region. Except for the *Eco*RI site present in the LTR, restriction mapping revealed no differences between the AKR ecotropic and MCF 247 proviruses for at least 5.1 kb from the 5′ terminus. Heteroduplex analysis as well as hybridization experiments using a xenotropic *env*-specific DNA probe demonstrated the presence of sequences related to xenotropic *env* determinants located between 6.2 and 6.9 kb from the 5′ terminus. The second clone represented a defective provirus with a 2.0-kb deletion of *env* sequences and contained nonoverlapping LTR sequences at each end of the cloned DNA segment. The deleted MCF DNA clone proved to be an immensely useful reagent in heteroduplex analyses of proviral DNAs, since it generated readily recognizable circular struc-

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tures after annealing to proviruses containing complete LTRs. In addition, proviral DNAs which contained *env* sequences formed a characteristic deletion loop when hybridized to the defective MCF cloned DNA.

The nucleotide sequence of the LTRs in the two MCF 247 cloned DNAs was determined. An interesting difference between the two DNA clones was the presence of a 104-base-pair (bp) direct repeat in the LTR of deleted proviral DNA which was present as a single copy in the clone that was colinear with the intracellular AKR MCF 247 provirus.

MATERIALS AND METHODS

Cells, virus, and DNA. High-molecular-weight DNA was prepared as previously described (38) from a mink lung cell line (ATCC CCL-64) (15) chronically infected with AKR MCF 247 MuLV (5, 14). The cells were grown in a Dulbecco-Vogt modification of Eagle minimal essential medium supplemented with 10% heated fetal calf serum. Unintegrated viral DNA was isolated by the Hirt procedure (16) from CCL-64 cells infected by an overlay of AKR MCF 247 MuLV-infected mink cells. Twenty-four hours after infection, the cells were lysed, and DNA was harvested as described (17).

Restriction enzyme digestion, gel electrophoresis, and DNA hybridization. Restriction endonucleases were purchased from Bethesda Research Laboratories (Rockville, Md.) and New England Biolabs (Beverly, Mass.). Cloned DNA (0.2 μg) or 5 μg of unintegrated viral DNA was digested with 2 U of enzyme per μg of DNA under the buffer conditions specified by the manufacturer. To monitor the completeness of digestion, wild-type lambda phage DNA was added to a portion of the reaction mixture. For more than one cleavage, a restricted DNA sample was ethanol precipitated, and the pellet was washed in 80% ethanol, air dried, and suspended in the reaction buffer of the second enzyme.

Digested DNA was analyzed by overnight electrophoresis at 30 V on 0.6% agarose horizontal slab gels (20 by 25 cm) as previously described (17, 26). The standard size marker mixture consisted of DNA fragments ranging from 23.5 to 0.07 kb and was prepared by combining the products of *Hin*dIII plus *Sma*I-cleaved lambda and *Hpa*II plus *Hae*III-digested ϕX174 replicative-form DNAs. The DNA was visualized by UV transillumination of ethidium bromide-stained gels and transferred to nitrocellulose filters as described by Southern (37). The filters were baked at 80°C for 2 h, preincubated, hybridized, and washed as previously described (17).

Molecular cloning of AKR MCF 247 provirus. *Eco*RI-restricted high-molecular-weight DNA obtained from CCL-64 cells chronically infected with AKR MCF 247 MuLV was mixed with λgtWES.λB vector arms, ligated with T4 DNA ligase, packaged in vitro into infectious phage particles, and plated on *Escherichia coli* DP50 *supF* as previously described (13). Recombinant phage plaques were screened (1), using ^{32}P-labeled AKR ecotropic MuLV cDNA. Positive plaques were subcloned and propagated, and DNA was isolated as described (13). The MuLV-reactive DNA inserts from the two lambda clones, designated λMCF 1 and λMCF 2, were released by *Eco*RI cleavage and ligated to *Eco*RI-digested pBR322 DNA by incubation with T4 DNA ligase. *E. coli* K-12 strain HB101 cells were transformed by the ligation mixture, and the recombinant plasmids containing the MCF 1 and MCF 2 DNA inserts, designated pMCF 1 and pMCF 2, respectively, were identified (12) and propagated as previously described (13).

Preparation of DNA probes. Single-stranded ^{32}P-labeled AKR ecotropic MuLV cDNA probe was synthesized as previously described (13). The MuLV LTR DNA probe represents the 600-bp *Kpn*I fragment from cloned Harvey sarcoma proviral DNA (13) isolated by elution from an agarose gel as described previously (24). The construction of the recombinant plasmid clone consisting of the 400-bp ecotropic-specific *env* segment (3) from a cloned infectious AKR ecotropic MuLV DNA (21) has been previously described. The xenotropic *env*-specific recombinant DNA clone (pX_{env}) consists of a 500-bp segment, derived from a partial clone of NFS-Th-1 xenotropic provirus, containing sequences located between 6.2 and 6.7 kb from the 5′ end of the viral genome (2a). The DNAs were labeled by nick translation (23) and had specific activities of 6×10^6 to 13×10^6 cpm/μg.

Cloned retroviral DNAs used in heteroduplex analyses. The ecotropic proviral DNA used as a standard in heteroduplex studies consisted of an 11.0-kb *Hin*dIII-*Eco*RI segment derived from λ AKR 623 (21). This DNA segment contains a complete infectious ecotropic provirus (8.8 kb) as well as 5′ (1.7 kb) and 3′ (0.5 kb) flanking mouse cellular DNA inserted into *Hin*dIII plus *Eco*RI-digested pBR322 DNA (pAKR 623). pAKR $623_{3'\text{-}B/R}$ is a recombinant plasmid containing the 3′-terminal 2.35-kb *Bam*HI-*Eco*RI segment of pAKR 623 DNA. The proviral DNA sequences present in pAKR $623_{3'\text{-}B/R}$ DNA extend from the *Bam*HI site at map position 7.0 kb (see Fig. 2) to the 3′ terminus (including the 3′ LTR).

DNA was mounted for electron microscopy by the formamide procedure essentially as described by Davis et al. (7). Grids were examined in a Siemens Elmiskop 101 electron microscope at 40-kV accelerating voltage. Electron micrographs were taken on Kodak electron image plates at magnifications of 4,000 to 6,000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam, catalog no. 1,000), and contour lengths were measured with a Numonics graphics calculator interfaced to a Wang 2200 computer.

DNA sequence and analysis. The sequence was determined by the chemical method of Maxam and Gilbert (25). Computer analysis was performed by using the program of Queen and Korn (31).

RESULTS

Molecular cloning of proviral DNA from mink cells infected with AKR MCF 247 MuLV. When molecular cloning studies were initiated more than 2 years ago, it was assumed that AKR MCF proviral DNAs, like AKR ecotropic proviruses, might not contain an internal *Eco*RI cleavage site and could be cloned by using the λgtWES.λB vector. DNA was therefore pre-

pared from mink cells chronically infected with AKR MCF 247 MuLV, digested with *Eco*RI, and ligated to the purified *Eco*RI arms of λgtWES.λB DNA as described in Materials and Methods. After in vitro packaging (13), recombinant phage plaques containing MuLV-reactive DNA were identified (1) by using ^{32}P-labeled AKR ecotropic MuLV cDNA. Of the 10^5 plaques screened, two (λMCF 1 and λMCF 2) hybridized to the cDNA probe and were found to contain DNA inserts 6.8 and 6.9 kb, respectively, in size. The relationship of the infected mink cell DNA inserts in λMCF 1 and λMCF 2 to the AKR MCF 247 provirus was determined by hybridizing ^{32}P-labeled AKR ecotropic MuLV cDNA to MCF 247-infected cellular DNA. A single 8.8-kb DNA species (Fig. 1, lane a), representing the unintegrated linear form of AKR MCF 247 proviral DNA, was detected which annealed to the cDNA probe. *Eco*RI digestion of the 8.8-kb linear proviral DNA generated 6.8- and 1.5-kb fragments which hybridized to the ^{32}P-labeled AKR MuLV cDNA probe (Fig. 1, lane b) as well as a 0.5-kb cleavage product detected in the autoradiogram only upon longer exposure (Fig. 1, lane e). Since these *Eco*RI fragments also annealed to the ^{32}P-labeled LTR DNA probe (data not shown), the results were compatible with *Eco*RI sites being located in the *env* and the LTR regions of AKR MCF 247 proviral DNA (Fig. 1, bottom), as has been previously suggested (3a, 29). *Eco*RI cleavage of λMCF 1 DNA yielded a 6.8-kb DNA segment (Fig. 1, lane c) which comigrated with the 6.8-kb *Eco*RI digestion product of the MCF 247 provirus (Fig. 1, lane b), whereas the insert present in λMCF 2 DNA (Fig. 1, lane d) was slightly larger (6.9 kb) in size. λMCF 1 and λMCF 2 DNAs were digested with *Eco*RI, and the proviral DNA segments were inserted into the *Eco*RI site of pBR322 DNA as described in Materials and Methods, generating recombinant plasmids designated pMCF 1 and pMCF 2, respectively.

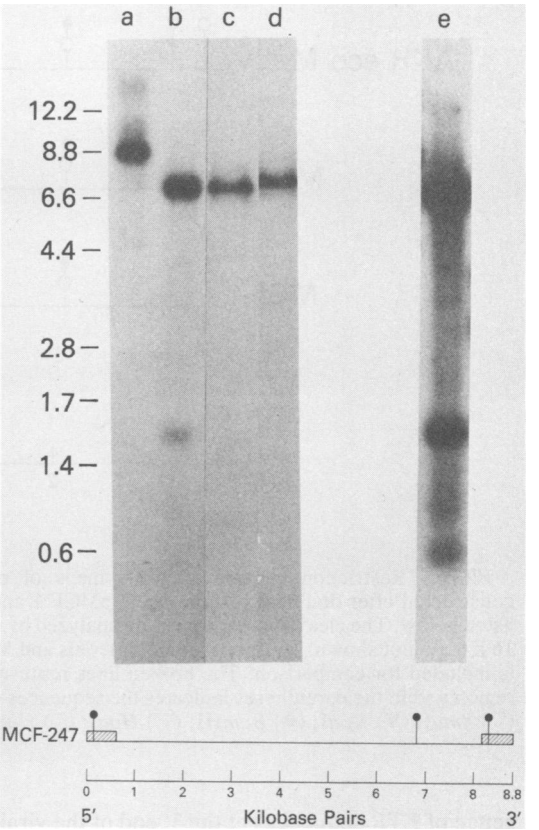


FIG. 1. Comparison of pMCF 1 and pMCF 2 DNAs with MCF 247 MuLV proviral DNA. DNA was prepared from mink cells chronically infected with MCF 247 MuLV as described in Materials and Methods. Uncleaved (lane a) and *Eco*RI-digested (lane b) infected mink cell DNA (5 μg each) and *Eco*RI-cleaved pMCF 1 (lane c) and pMCF 2 (lane d) DNAs (0.2 μg each) were subjected to electrophoresis on a 0.6% horizontal agarose slab gel at 30 V for 16 h. After transfer to a nitrocellulose membrane, the DNA was hybridized to ^{32}P-labeled AKR ecotropic MuLV cDNA (5×10^6 cpm) and washed as described in Materials and Methods. Lane e is a 1-week exposure of lane b (24-h exposure) for the visualization of the 0.5-kb DNA fragment. The location of the *Eco*RI restriction sites in MCF 247 proviral DNA is diagramed at the bottom. The numbers represent the size (in kilobases) of some of the DNA fragments present in the standard marker mixture described in Materials and Methods.

Characterization of pMCF 1 DNA. By several criteria, the DNA insert present in pMCF 1 was shown to be the 6.8-kb AKR MCF 247 proviral DNA segment extending from the *Eco*RI site located in the 5′ LTR to the *Eco*RI site situated in the *env* region. The restriction endonuclease maps of MCF 1 and AKR ecotropic proviral DNAs were indistinguishable for 5.1 kb from the 5′ terminus except for the *Eco*RI site in the LTR of MCF 1 DNA (Fig. 2). MCF 1 proviral DNA contained unique restriction sites mapping at 6.1 (*Pvu*II), 6.2 (*Bam*HI), and 6.9 (*Eco*RI) kb which were absent in the AKR ecotropic provirus. Conversely, several restriction sites located between 5.8 and 6.9 kb in the ecotropic proviral DNA were not detected in pMCF 1 DNA (Fig. 2). The *Pvu*II and *Eco*RI sites have been located at similar positions in the genome of several xenotropic MuLV DNAs (2a, 3a).

Blot hybridization analysis of *Eco*RI plus *Sma*I-cleaved MCF 1 DNA demonstrated the reaction of only the 5′ 0.45- and 4.6-kb fragments with the LTR probe, indicating the ab-

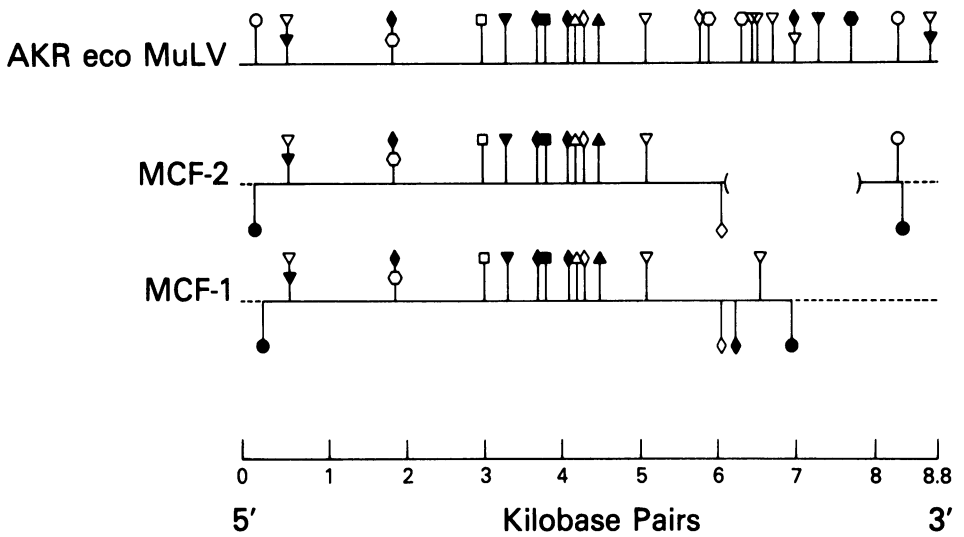


FIG. 2. Restriction enzyme cleavage maps of pMCF 1 and pMCF 2 DNAs. Restriction maps were constructed after double digestion of the pMCF 1 and pMCF 2 proviral DNAs, using the restriction enzymes listed below. The cleavage products were analyzed by electrophoresis on 0.6% horizontal agarose gels at 30 V for 16 h (data not shown), as described in Materials and Methods. The AKR ecotropic MuLV DNA restriction map is included for comparison. The broken lines represent the sequences not present in the proviral DNAs. The region within the parentheses indicates the sequences deleted from pMCF 2 DNA. Symbols: (●) *Eco*RI; (○) *Pst*I; (▽) *Sma*I; (▼) *Kpn*I; (◆) *Bam*HI; (○) *Hpa*I; (□) *Hin*dIII; (●) *Xba*I; (■) *Sac*I; (△) *Sal*I; (◇) *Pvu*II; (▲) *Xho*I.

sence of LTR sequences at the 3′ end of the viral DNA insert (Fig. 3, lane c). To determine the envelope specificity in MCF 1 DNA, *Bam*HI plus *Eco*RI cleavage products were annealed to a ^{32}P-labeled probe specific for ecotropic envelope sequences (3) (Fig. 4, panel II, lane 3). No hybridization was detected with the virus-specific DNA segments, whereas fragments A and E reacted due to the pBR322 DNA sequences present in the labeled probe. In the case of *Bam*HI plus *Eco*RI-cleaved pAKR 623 ecotropic MuLV DNA (pAKR eco) (Fig. 4, panel II, lane 1), in addition to fragments A and C, which contained pBR322 DNA sequences, hybridization to the 2.9-kb cleavage product (fragment B), located in the *env* region, was observed due to the derivation of the ecotropic *env*-specific probe from this DNA segment (3). The positions of the reactive fragments on the viral genome are shown at the bottom of Fig. 4.

We have recently constructed a recombinant plasmid (pX_{env}) containing a 500-bp DNA segment subcloned from the *env* region of xenotropic proviral DNA, which does not hybridize to ecotropic MuLV proviral DNA but does anneal to representative xenotropic and four different MCF proviruses (2a). ^{32}P-labeled pX_{env} DNA annealed to the 6.8-kb *Eco*RI-cleaved MCF 1 DNA segment (data not shown). The xenotropic *env*-specific DNA probe also hybridized to the 700-bp *Bam*HI plus *Eco*RI fragment D of pMCF 1 DNA and not to the cloned AKR 623 ecotropic provirus (Fig. 4, panel III, lanes 3 and 1, respectively). Hybridization to the 4.1-kb cleavage product (fragment A) in this experiment represents annealing of plasmid DNA sequences present in labeled pX_{env} DNA to the pBR322 DNA component of pMCF 1 DNA. No reaction of the xenotropic *env* probe with the 375-bp *Bam*HI plus *Eco*RI fragment (fragment E, Fig. 4) of pBR322 DNA was observed, since this segment of the plasmic DNA was eliminated during the construction of pX_{env} DNA (2a). The MCF 247-infected mink cell DNA contained the same *env* specificities as the MCF 1 DNA; hybridization was observed to the pX_{env} DNA probe but not to labeled AKR ecotropic *env*-specific DNA (data not shown).

Electron microscopic analysis of heteroduplex molecules formed by using MCF 1 and cloned AKR ecotropic proviral DNAs further supported the relatedness shown in Fig. 2. Heteroduplex molecules consisted of a 6.2-kb duplex segment showing no visible areas of mismatch and a small (0.6-kb) terminal heterology branch presumably representing xenotropic *env*-related sequences located at the 3′ end of MCF 1 DNA ("c" in Fig. 5A).

Characterization of pMCF 2 DNA. A restriction enzyme cleavage map of MCF 2 proviral

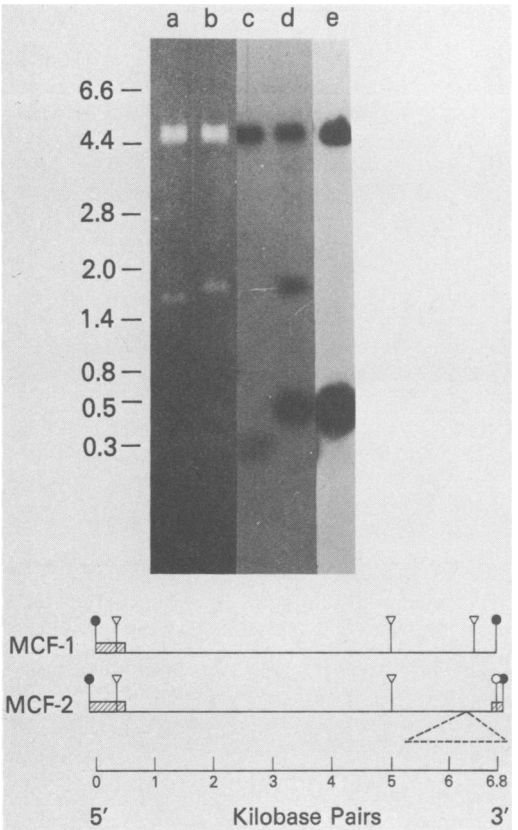


FIG. 3. Characterization of LTR sequences in pMCF 1 and pMCF 2 proviral DNAs. pMCF 1 (lanes a and c) and pMCF 2 (lanes b and d) DNAs (0.2 μg each) were cleaved with *Eco*RI plus *Sma*I and electrophoresed through a 0.6% agarose gel at 30 V for 16 h as described in Materials and Methods. After staining with ethidium bromide (lanes a and b), the DNA was transferred to nitrocellulose membranes and hybridized to the ^{32}P-labeled LTR DNA probe (2×10^6 cpm) (lanes c and d) as described in Materials and Methods. An autoradiogram showing the reaction of the ^{32}P-labeled LTR probe to *Eco*RI plus *Sma*I plus *Pst*I-digested pMCF 2 DNA is presented in lane e. The maps at the bottom show the locations of the *Eco*RI (●), *Pst*I (○), and *Sma*I (▽) restriction sites and the LTR (▨). The broken triangle represents the deletion in MCF 2 DNA. The numbers at the left indicate the size (in kilobases) of some of the DNA fragments present in the standard marker mixture described in Materials and Methods.

DNA was prepared by carrying out a series of double digestions with the enzymes listed in Fig. 2. Except for differences in the 5′ LTRs (as discussed below), MCF 2 and MCF 1 viral DNAs were indistinguishable from one another between the *Eco*RI site located at the 5′ terminus and the *Pvu*II site which mapped at 6.1 kb (Fig. 2). Beyond this point, MCF 2 proviral DNA lacked the 6.3-kb *Bam*HI, the 6.5-kb *Sma*I, and the 6.9-kb *Eco*RI restriction sites present in MCF 1 DNA and contained a unique *Pst*I site located at 6.9 kb and an *Eco*RI site mapping at 7.0 kb (Fig. 2). pMCF 2 DNA failed

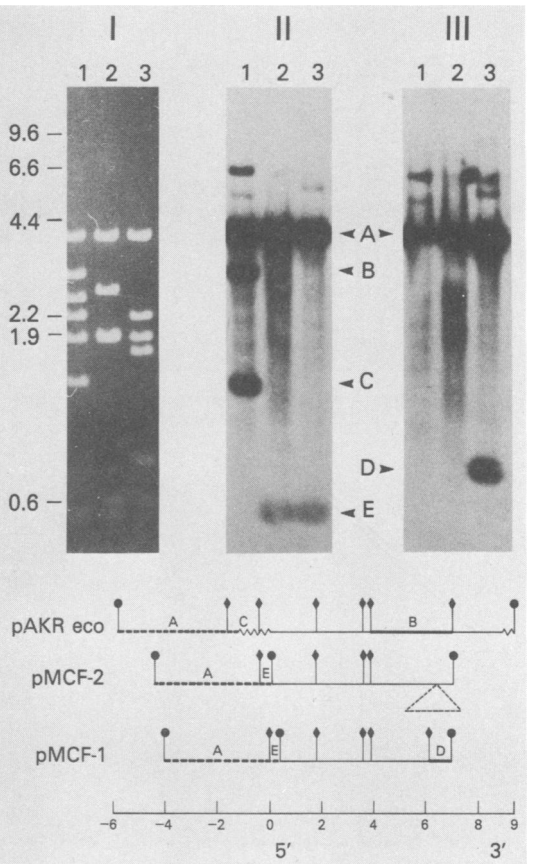


FIG. 4. *env* reactivities of MCF 1 and MCF 2 cloned DNAs. pAKR (lane 1), pMCF 2 (lane 2), and pMCF 1 (lane 3) DNAs (0.3 μg each) were digested with *Bam*HI plus *Eco*RI and subjected to 0.6% horizontal agarose gel electrophoresis at 30 V for 16 h as described in Materials and Methods. The DNA was transferred to nitrocellulose filters and hybridized to ^{32}P-labeled DNA probes (2×10^6 cpm), and the membranes were washed and exposed to film as described in Materials and Methods. Panel I shows a UV-fluorescence photograph of the ethidium bromide-stained digests. The autoradiograms using ^{32}P-labeled ecotropic *env*-specific and xenotropic *env*-specific DNA probes are shown in panels II and III, respectively. The size (in kilobases) of some standard marker fragments are indicated at the left. The position of the *Bam*HI plus *Eco*RI fragments (A to E) is indicated on the maps of the three recombinant plasmids at the bottom of the figure. The heavy broken line represents pBR322 DNA; the dashed triangle indicates sequences deleted from MCF 2 DNA; the wavy line indicates flanking mouse cellular DNA; and the heavy solid line indicates segments reactive with the *env*-specific DNA probes.

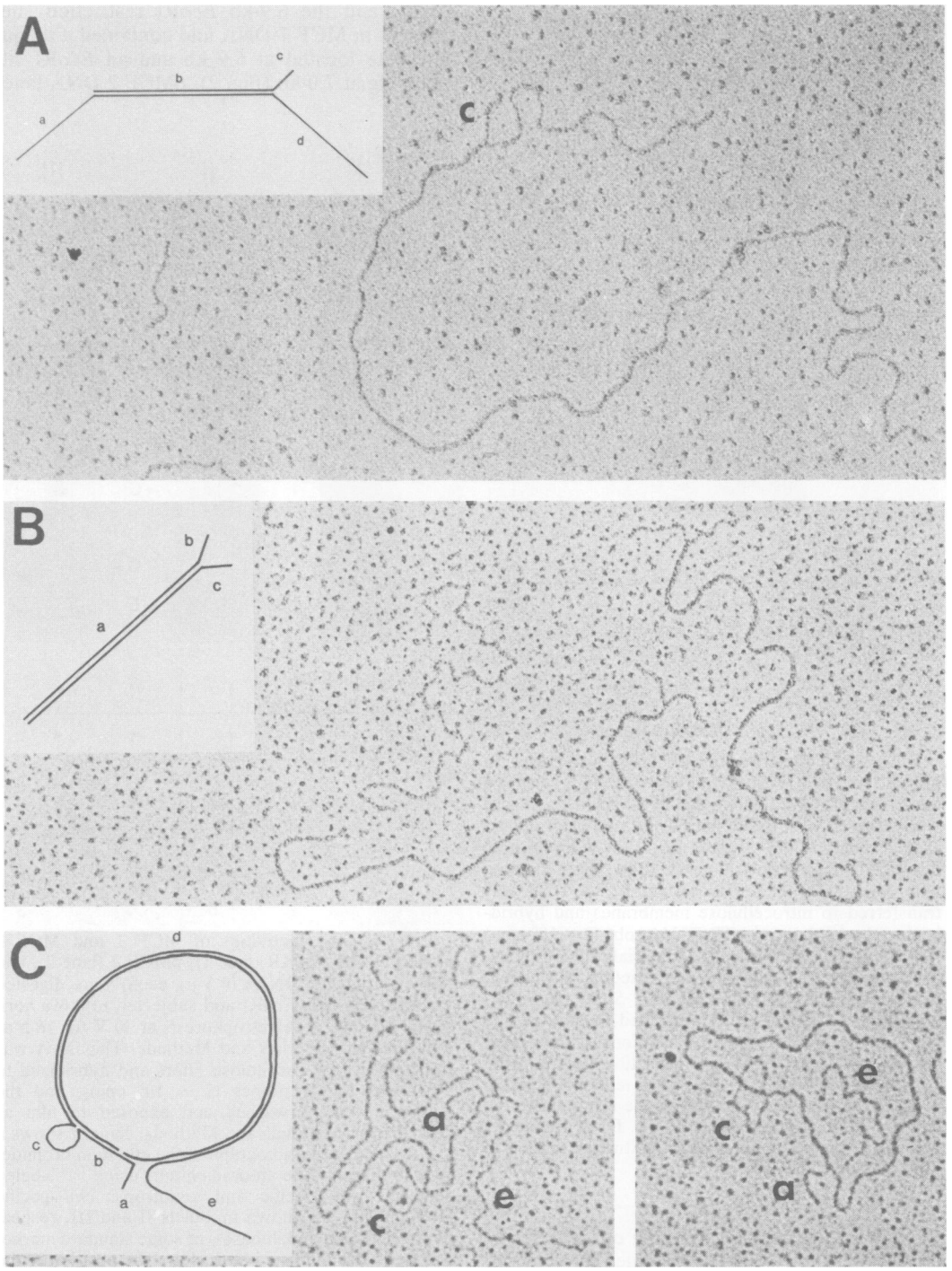


FIG. 5. Electron micrographs of representative heteroduplex molecules formed between pMCF 1 and pAKR proviral DNAs (panel A), pMCF 1 and pMCF 2 DNAs (panel B), and pMCF 2 and pAKR DNAs (panel C). Heteroduplexes were formed by mixing equal quantities (5 to 10 μg) of each *Eco*RI-digested DNA, denaturing with alkali, and renaturing for 2 h at 35°C in 50% formamide containing 0.1 M Tris, pH 8.5, and 1 mM EDTA. Measurements of various segments are expressed in kilobases (± standard deviation), using internal calibration standards of known molecular weight, and represent mean values. Panel A (n = 28 molecules): a, 6.6 ± 0.9; b, 6.2 ± 0.6; c, 0.6 ± 0.05; and d, 3.4 ± 0.2. Panel B (n = 25 molecules): a, 6.1 ± 0.2; b, 0.6 ± 0.07; and c, 0.5 ± 0.07. Panel C (n = 45 molecules): a, 0.6 ± 0.1 kb; b, 0.9 ± 0.1; c, 1.8 ± 0.2; d, 5.6 ± 0.1; and e, 7.5 ± 0.5.

to hybridize to either the ecotropic or xenotropic envelope-specific DNA probes (Fig. 4, lanes 2; panels II and III, respectively). When MCF 2 DNA was heteroduplexed with MCF 1 DNA, molecules containing 6.1 kb of double-stranded DNA with a nonhomologous terminal fork structure measuring approximately 0.5 kb were observed (Fig. 5B). This region of heterology most likely involves sequences located at the 3′ end of MCF 2 DNA, since this DNA segment failed to anneal to envelope-specific DNA probes (Fig. 4) and differed from MCF 1 DNA in its restriction enzyme map (Fig. 2).

When pMCF 2 DNA was double digested with *Eco*RI plus *Sma*I and hybridized to the LTR probe, reaction was observed with all three restriction fragments, including the 1.6-kb 3′ cleavage product (Fig. 3, lane d). This result clearly indicated that the 6.9-kb *Eco*RI MCF 2 DNA segment contained LTR sequences at each end, as diagramed at the bottom of Fig. 3. The organization of LTR sequences located at the 3′ terminus of MCF 2 proviral DNA was further evaluated by digesting pMCF 2 DNA with *Pst*I in addition to *Eco*RI plus *Sma*I, followed by hybridization with the LTR probe. Annealing of labeled LTR DNA to the 1.6-kb *Sma*I plus *Eco*RI fragment was abolished after cleavage with *Pst*I (Fig. 3, lane e), implying that LTR sequences extended from the *Eco*RI site at the 3′ terminus of MCF 2 DNA to a region in the general vicinity of the *Pst*I site. The location of the *Eco*RI and *Pst*I sites within the LTR as shown in Fig. 3 was substantiated by nucleotide sequence analysis (see Fig. 8). MCF 2 viral DNA therefore represented a deleted MCF provirus, 6.9 kb in size, which was indistinguishable from MCF 1 DNA (and presumably the AKR MCF 247 provirus) in its first 6.1 kb and contained LTR sequences at each end.

Electron microscopy of heteroduplex molecules involving MCF 2 proviral DNA. Analysis of heteroduplex structures formed between MCF 2 proviral DNA and a cloned AKR ecotropic provirus could directly confirm the existence as well as the position of the putative deletion in MCF 2 proviral DNA predicted from restriction enzyme mapping and blot-hybridization studies. Quite unexpectedly, instead of linear duplex structures containing a deletion loop approximately 6.0 kb from the 5′ end, double-stranded circular molecules with a single deletion loop and two single-stranded tails of dissimilar lengths were observed (Fig. 5C) at high frequency. No single-stranded circles were found. In these experiments, pAKR 623 DNA was linearized by *Eco*RI digestion and annealed to pMCF 2 DNA which had been previously cleaved with *Eco*RI, releasing the 6.9-kb MCF 2 proviral DNA. Since the AKR 623 DNA insert has been shown to contain 0.5 kb of mouse DNA sequences which extend from the 3′ LTR of the cloned provirus to a cellular *Eco*RI site (21), the short single-stranded segment seen in the heteroduplex ("a" in Fig. 5C) gives the orientation of these flanking sequences. Similarly, the longer single strand of DNA attached to the double-stranded circle ("e" in Fig. 5C) represents mouse DNA sequences present in the AKR 623 clone which abut the 5′ LTR and extend 1.7 kb to a *Hin*dIII site, where they are joined to pBR322 DNA. Contour length measurements of circular duplex molecules resulting from annealing of AKR proviral DNA with MCF 2 viral DNA indicate that a 1.8-kb deletion loop ("c" in Fig. 5C) maps approximately 0.9 kb from the 3′ terminus of the cloned AKR provirus ("b" in Fig. 5C).

The proximity of the attachment sites of the single-stranded flanking mouse DNA segments abutting the 5′ and 3′ LTRs of AKR MuLV DNA in the circular duplex molecules shown in Fig. 5C strongly suggested that circularization was being effected by the annealing of an LTR segment of AKR 623 DNA with the homologous LTR sequences present at each end of MCF 2 DNA. To determine whether a single LTR could effect circularization, *Eco*RI-cleaved MCF 2 DNA was annealed to a recombinant plasmid containing only the 2.35-kb *Bam*HI plus *Eco*RI 3′-terminal segment of AKR 623 cloned DNA which included the 3′ LTR of the AKR ecotropic provirus (designated as pAKR $623_{3'\text{-}B/R}$). MCF 2 DNA formed a partially double-stranded circular molecule with this subgenomic fragment of the ecotropic proviral DNA (Fig. 6A). In this particular case, the homologous LTR regions as well as adjacent proviral DNA sequences which encode p15E-R protein (19, 39, 40) (see below) formed a 1.1-kb duplex segment ("b" in Fig. 6A). The long single-stranded tail ("a" in Fig. 6A) that extended from the circle contained AKR ecotropic *env* and plasmid DNA sequences which did not anneal to MCF 2 DNA. The 0.6-kb single-stranded DNA segment ("c" in Fig. 6A) represented mouse cellular DNA sequences which flanked the 3′ LTR of cloned AKR 623 DNA. Since circular duplex molecules were also formed when a subgenomic DNA segment containing sequences from the *gag-pol* region along with the 5′ LTR was hybridized to MCF 2 DNA (data not shown), we concluded that only the LTR sequences were responsible for the formation of the circular structures. The unique terminal structure of the *Eco*RI-cleaved MCF 2 DNA segment, which contained nonoverlapping LTR sequences at either end of the same molecule, most likely effected circularization of the AKR proviral DNA by a mechanism illustrated in Fig. 7. This model predicts that any

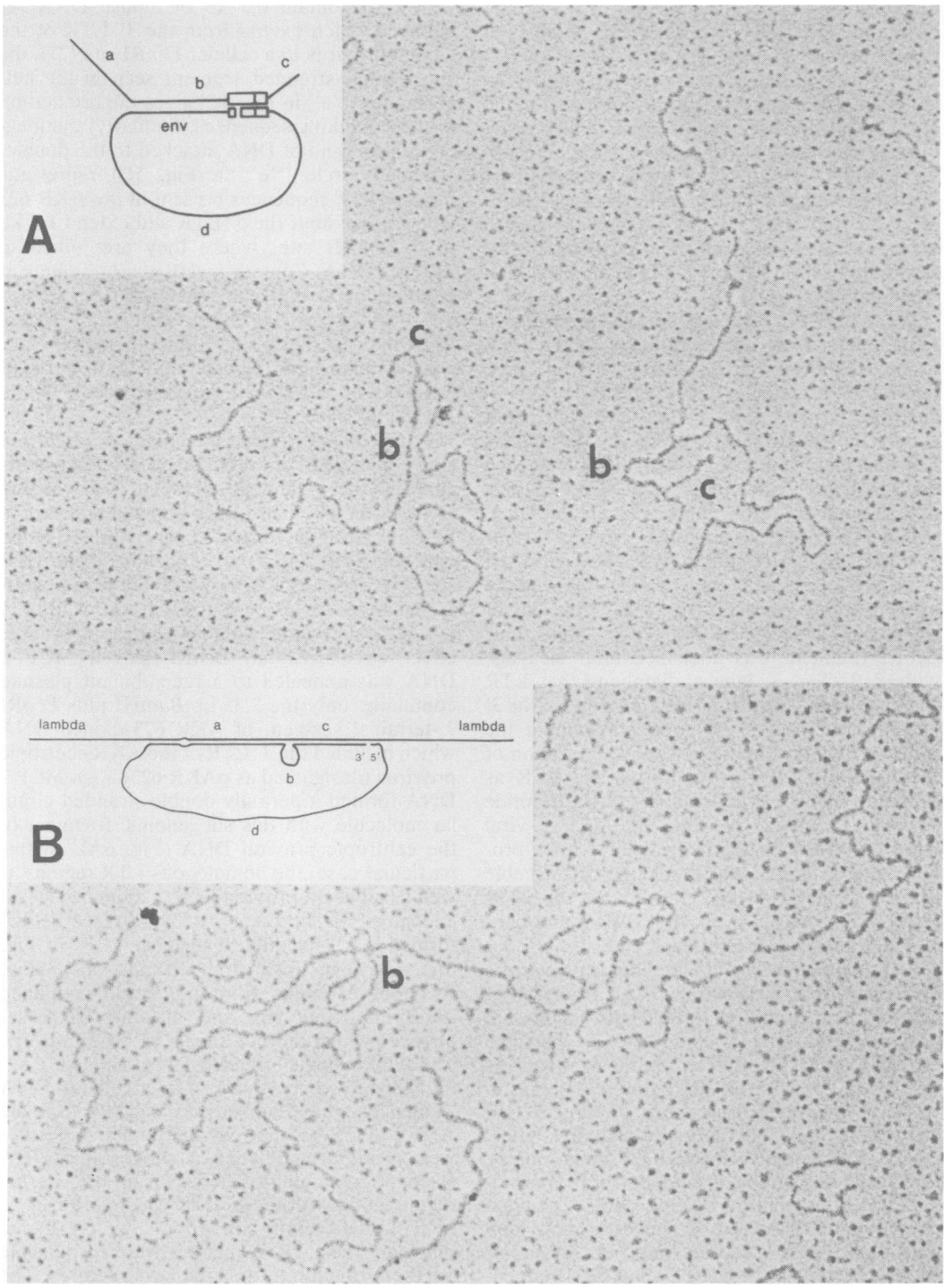


FIG. 6. Electron micrographs depicting circular heteroduplex molecules involving pMCF 2 DNA. The structures observed by annealing *Eco*RI-digested pMCF 2 DNA with either uncleaved pAKR $623_{3'\text{-}B/R}$ DNA (panel A) or an intact lambda recombinant DNA clone containing endogenous MuLV-related sequences (Khan et al., in preparation) (panel B). The conditions for the reaction mixture are as described in Fig. 5. Measurements of various segments are expressed as in Fig. 5. Panel A (n = 32 molecules): a, 4.9 ± 0.2; b, 1.1 ± 0.06; c, 0.6 ± 0.09; and d, 5.6 ± 0.3. Panel B (n = 10 molecules): a, 1.5 ± 0.2; b, 2.0 ± 0.2; and c, 1.4 ± 0.4.

DNA molecule containing at least a single copy of an LTR related to MCF 2 (AKR MCF 247) proviral DNA will form circular structures after annealing to MCF 2 DNA. The model presented in Fig. 7 also predicts that proviruses lacking certain LTR sequences will not form circles with MCF 2 DNA. This proved to be the case with MCF 1 DNA, which lacked the 78 nucleotides that preceded the *Eco*RI site in the LTR segment (see the sequence below) and thereby failed to generate circular structures after hybridization to MCF 2 DNA (Fig. 5B).

To test the potential usefulness of MCF 2 DNA as a reagent for identifying specific proviral DNA segments by electron microscopy, heteroduplex molecules were formed by annealing MCF 2 DNA to an MuLV-reactive clone isolated from a BALB/c mouse DNA library (A. S. Khan et al., manuscript in preparation). In this experiment, intact recombinant lambda phage DNA, containing a DNA insert which annealed to an LTR and a variety of other MuLV subgenomic DNA probes, was denatured and hybridized to *Eco*RI-cleaved MCF 2 DNA. A partially double-stranded circle containing a 2.0-kb deletion loop ("b" in Fig. 6B) and two long single-stranded tails (lambda DNA arms) was visualized. On the basis of these heteroduplex results, we concluded that this clone contained an LTR (circle formation) as well as retroviral *env* sequences (deletion loop) mapping 6.2 to 8.0 kb from the 5′ terminus. Restriction enzyme mapping and blot-hybridization analyses using subgenomic MuLV DNA probes confirmed this prediction and indicated that the BALB/c clone contained 4.0-kb MuLV-reactive DNA sequences (5.0 kb from contour length measurements) extending in the 5′ direction from the 3′ terminus (Khan et al., manuscript in preparation).

Sequencing of MCF 1 and MCF 2 DNAs. Nucleotide sequencing of the termini of MCF 2 DNA confirmed the results of hybridization experiments (Fig. 3, lanes d and e) which indicated the presence of LTR sequences at each end of cloned MCF 2 proviral DNA. Figure 8A shows the nucleotide sequence of the LTR segments of MCF 1 and MCF 2 DNAs. The sequence of the LTR of Moloney murine sarcoma (MoMSV) provirus (9) is included for comparison. The 5′ terminus of MCF 2 DNA begins at the *Eco*RI site and extends 550 nucleotides to the end of the LTR region. Conversely, the 3′ end of MCF 2 DNA also ends at the *Eco*RI site located in the LTR and contains 79 nucleotides of the 3′ LTR. We have assumed that the LTR sequences located at both termini of MCF 2 proviral DNA constitute a complete, nonoverlapping, but permuted LTR unit and have presented its nucleotide sequence in the conventional manner as the "reconstructed" MCF 2 LTR in Fig. 8A. This assumption was supported by the identification

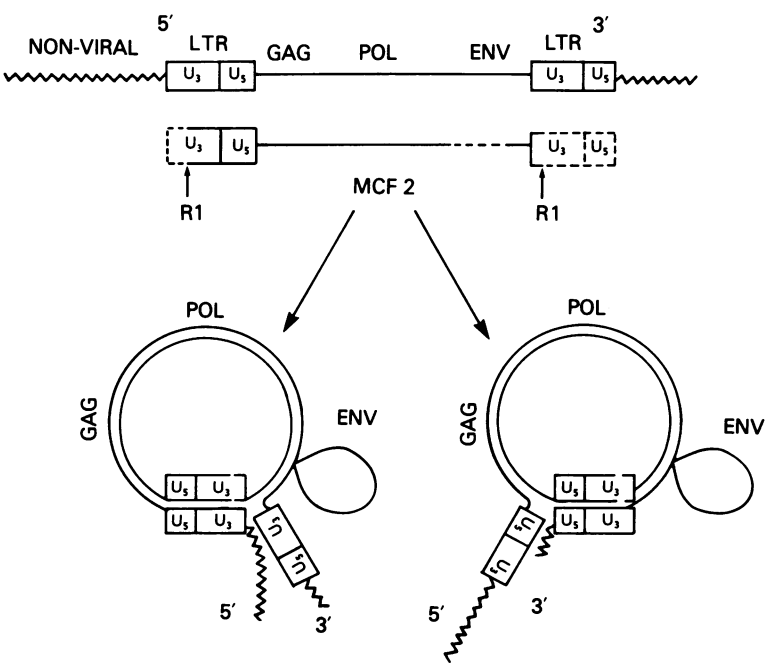
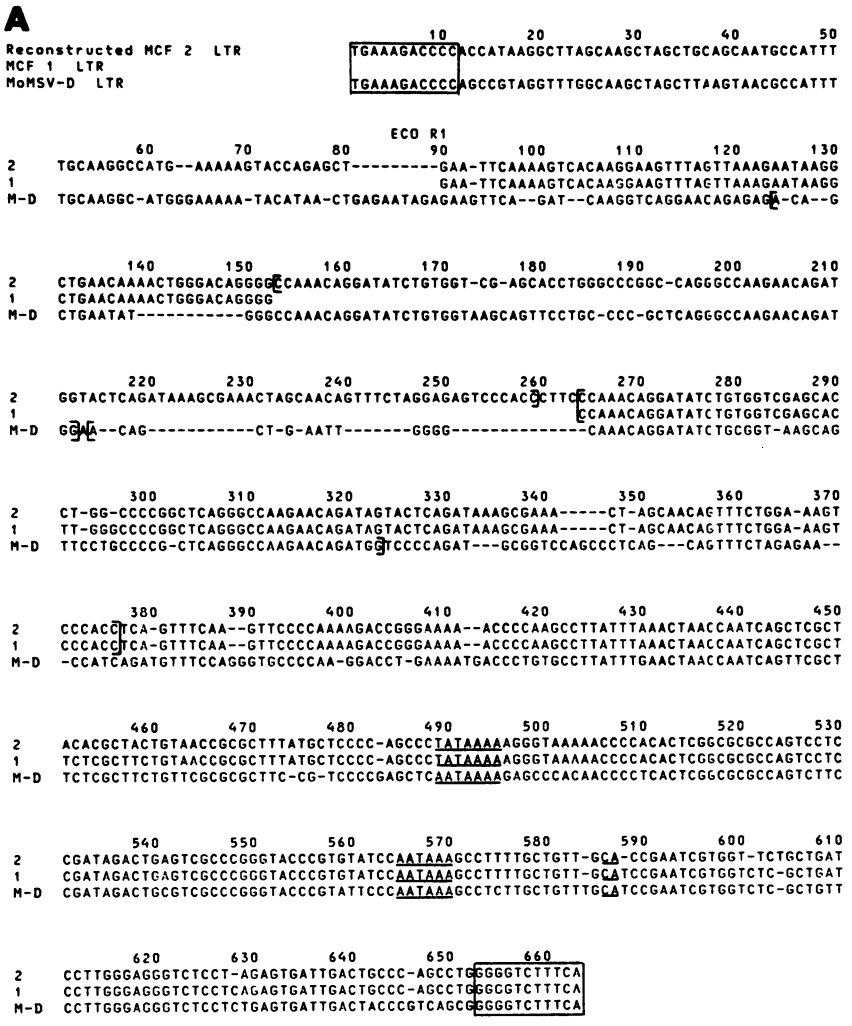


FIG. 7. Model visualizing how MCF 2 proviral DNA might effect the circularization of a retroviral DNA segment containing a complete LTR.


A
Reconstructed MCF 2 LTR
MCF 1 LTR
MoMSV-D LTR
ECO R1
B
MCF 2 3'
MoMuLV 3'
MCF
M
MCF
M
MCF
M

of an 11-bp inverted DNA segment present near both ends of MCF 2 DNA which was identical to the inverted terminal repeats that demarcate the boundary between the LTR and flanking cellular or adjacent proviral DNA sequences described for the MoMSV provirus (9, 34). Like MCF 2 DNA, the 5′ terminus of MCF 1 DNA begins at the *Eco*RI site located in the 5′ LTR of the MCF 247 provirus (Fig. 1, bottom), but extends only 444 nucleotides to the end of the LTR (Fig. 8A). The nucleotide sequences of MCF 1 and MCF 2 LTRs are very similar (total of 10 mismatched bases) except for the occurrence of a 104-bp direct repeat in the case of MCF 2 DNA, beginning at position 152, and the 4-bp intervening sequence separating each repeating unit (Fig. 8A). The smaller-sized LTR of MCF 1 DNA could also be predicted from the hybridization experiment depicted in Fig. 3, which shows that the 5′-terminal *Eco*RI plus *Sma*I fragment of MCF 1 DNA is about 100 nucleotides shorter (lane c) than the corresponding fragment derived from MCF 2 proviral DNA (lane d). We have arbitrarily aligned the single copy of the reiterated component of the direct repeat present in MCF 1 proviral DNA with the second repeating unit of MCF 2 DNA because fewer "errors" (two versus six) were generated by such an arrangement.

A comparison of the LTRs of MCF and MoMSV proviral DNAs demonstrated the conservation of previously identified functionally significant nucleotide sequences (shown in Fig. 8A) between the two retroviral DNAs. These included the 11-bp inverted terminal repeats, the polyadenylation signal located at positions 565 to 570, preceding the dinucleotide C-A in position 586 (which is the preferred site for polyadenylation) (30), and the putative promoter-like sequence (44) at positions 489 to 496. Apart from these highly conserved sequences, extensive nucleotide heterogeneity (about 33%) was observed in the U_3 region (which contains bases uniquely located at the 3′ end of the viral RNA), whereas only 13% nucleotide dissimilarity was noted in the U_5 region (which contains unique sequences at the 5′ end of the viral RNA). A notable difference in the nucleotide structure was the 104-bp direct repeat present in MCF 2 DNA, whereas MoMSV proviral LTR contained a 73-bp direct repeat. The greater heterogeneity in the U_3 region is also reflected by the presence of unique restriction sites in the LTR of MCF and MoMSV DNAs; *Eco*RI and *Pst*I restriction sites in the LTR of MCF DNA were absent in the LTR of MoMSV DNA, whereas several restriction sites mapped in the LTR region of the latter DNA (9, 34) were not present in the MCF provirus. The variability in the U_3 region has also been shown for several avian retroviral DNAs (18, 41). In contrast, the nucleotide sequence in the U_5 region was relatively conserved.

In addition to the 79 nucleotides comprising the partial LTR, the 3′ end of MCF 2 DNA contained viral sequences between the LTR and the deletion loop which formed a duplex structure in some heteroduplex mapping experiments (Fig. 5C and 6). The nucleotide sequence of a 162-bp segment located adjacent to the 3′ LTR of MCF 2 proviral DNA (designated as MCF 2 3′ in Fig. 8B) enabled us to determine the amino acid sequence which is related to that reported for the protein encoded by the *R* gene in Mo-MuLV (designated as MoMuLV 3′ in Fig. 8B) by Sutcliffe et al. (39, 40). The differences in the predicted amino acid sequences between MCF 2 and MoMuLV DNAs, underlined in Fig. 8B, were mainly localized near the carboxy terminus. The dissimilarity in the protein sequence was largely due to 5 amino acids encoded by a stretch of 15 nucleotides abutting the 3′ LTR which was absent in MoMuLV.

DISCUSSION

In this paper we have described the characterization of two proviruses isolated by shotgun cloning of *Eco*RI-restricted DNA obtained from AKR MCF 247 MuLV-infected mink cells. One of the clones (λMCF 1) contained DNA that was indistinguishable from the 6.8-kb DNA segment extending from the *Eco*RI site located in the 5′ LTR to the *Eco*RI site in the *env* region of the

FIG. 8. (A) Nucleotide sequences of MCF 1 and "reconstructed" MCF 2 proviral LTRs are compared with the LTR sequence of MoMSV DNA published by Dhar et al. (9) (MoMSV-D [M–D]). Bases are aligned to illustrate maximum homologies; dashes are inserted to bridge regions where no corresponding base occurs in one of the other sequences. Consecutive bases for a given LTR would be read by omitting dashes; numbering is an aid for reference and does not identify the actual base number. The inverted terminal repeats are boxed, internal direct repeat sequences in MCF 2 (104 bp) and MoMSV-D (73 bp) are enclosed in brackets, and functionally significant sequences are underlined. The nucleotide sequence of MCF 1 DNA begins at position 90; the blank region in MCF 1 DNA (positions 153 to 263) corresponds to the absence of duplicated sequences of the direct repeat present in MCF 2 DNA. (B) The 3′ end of the *env* genes of MCF 2 (MCF) and MoMuLV (M) (39, 40) are compared. Underlined are the amino acids encoded by MCF 2 which differ from those of MoMuLV. The sequences downstream from the coding region contain the origin of plus-strand DNA synthesis. The last nucleotide of the noncoding region precedes the first nucleotide of the 3′ LTR. Data are presented 5′ to 3′.

MCF 247 provirus. Except for the *Eco*RI site in the LTR, the restriction map of MCF 1 proviral DNA was identical to that of the AKR ecotropic provirus for at least 5.1 kb from the 5′ terminus. DNA sequences extending from map position 5.1 to 6.9 kb in MCF 1 provirus contained several restriction enzyme cleavage sites not present in AKR ecotropic MuLV DNA. Consistent with the presence of these new sites was the failure of the MCF 1 DNA to hybridize to the 500-bp ecotropic envelope-specific DNA probe derived from this region (6.5 to 7.0 kb from the 5′ terminus) (3) of the AKR ecotropic provirus. On the other hand, MCF 1 DNA hybridized to a xenotropic *env*-specific DNA probe containing sequences located between 6.2 and 6.7 kb from the 5′ end of the NFS-Th-1 xenotropic provirus (2a). The xenotropic envelope reactivity was mapped to the region between the 6.2-kb *Bam*HI site and the 6.9-kb *Eco*RI site on the MCF 1 provirus. MCF 1 (and presumably MCF 247) proviral DNA thus contains approximately 6.0 kb of sequences extending from the *Eco*RI site in the 5′ LTR that are indistinguishable from those present in ecotropic proviral DNA and which are joined to a 700-bp DNA segment abutting the 6.9-kb *Eco*RI site containing xenotropic envelope specificity. It should also be noted that the 3′ region of the MCF 1 DNA insert contains two restriction sites (the 6.1-kb *Pvu*II and the 6.9-kb *Eco*RI sites) that have been mapped in similar positions in several xenotropic proviral DNAs (2a, 3a). An analysis of the heteroduplex structures formed after annealing of the MCF 1 DNA with AKR ecotropic MuLV DNA revealed a 6.2-kb duplex and a 3′ 0.5-kb nonhomologous segment. This latter short region of heterology undoubtedly reflects the difference in the restriction enzyme cleavage sites and *env* specificities located between 6.0 and 6.9 kb on the ecotropic and MCF proviruses. The relatedness between MCF and AKR viral genomes shown in this study is consistent with previous reports involving T_1 oligonucleotide mapping analysis which demonstrated similarities in the *gag-pol* region and uniqueness in the *env* region between thymotropic and nonthymotropic MCFs (36) and AKR ecotropic MuLV genomes (22, 35). In addition, thymotropic MCFs (including MCF 247) contained a new MCF-specific T_1 oligonucleotide in the 3′-terminal sequences which corresponded to the LTR region in the proviral DNA (22). In this latter regard, a comparison between the nucleotide sequences present in the MCF proviral LTR (discussed below) and in the LTR of AKR ecotropic MuLV DNA (C. Van Beveren, E. Rands, S. K. Chattopadhyay, D. R. Lowy, and I. M. Verma, personal communication) revealed heterogeneity (about 20%) in the U_3 region of the LTRs; the U_5 region was identical except for a 4-bp mismatch.

The MCF 2 provirus, which was isolated from the same shotgun cloning experiment that yielded MCF 1 DNA, contained a deletion in the *env* region. This DNA probably represents a copy of a defective MuLV generated during the propagation of AKR MCF 247 in mink cells rather than an artifact of molecular cloning, since it is highly unlikely that an 8.8-kb partial *Eco*RI digestion product of the MCF 247 provirus (see Fig. 1) was inserted into the λ cloning vector during the initial phases of shotgun cloning with the subsequent loss, during propagation in *E. coli*, of a 2.0-kb segment containing the *Eco*RI site located in the *env* region. As described above, the MCF 2 proviral DNA has the potential for being an immensely useful reagent for heteroduplex experiments. Despite the absence of the 2.0-kb segment encoding the major portion of the MCF 247 envelope, MCF 2 DNA and its subgenomic derivatives have been used for the characterization of other cloned MuLV proviruses. In this latter regard, the ability of MCF 2 DNA to form circular heteroduplex molecules with cloned MuLV-related proviruses has greatly facilitated our identification of LTR regions in endogenous retroviral clones (Khan et al., in preparation). Furthermore, the presence of the 2.0-kb deletion in MCF 2 proviral DNA generates a characteristic deletion loop after its annealing to a provirus containing the envelope-coding region (Fig. 6B), thus allowing determination of the 5′ 3′ orientation of the DNA sequences with respect to the viral genome.

Although both MCF 1 and MCF 2 proviral DNAs have identical restriction enzyme cleavage maps extending 6.1 kb from their 5′ termini, a difference in size of their LTRs was observed by hybridization (Fig. 3, lanes c and d). The variation in the LTRs was resolved by nucleotide sequence analysis which demonstrated that the MCF 1 proviral DNA did not contain the 104-bp direct repeat present in the LTR of MCF 2 DNA (Fig. 8A). Apart from this difference, the nucleotide sequences of the LTRs in the MCF proviruses were nearly identical, with only 10 mispaired bases. An interesting feature of the direct repeat found in the LTR of MCF 2 DNA is that it is longer (104 versus 73 bp) and only partially homologous to the direct repeat in MoMSV described by Dhar et al. (9), the sequence of which is presented in Fig. 8A for comparison. The latter begins 117 bp from the 5′ end of the LTR (at position 124) and can be aligned with the direct repeat of MCF 2 proviral DNA for 51 and 48 bp within positions 153 to 212 and 264 to 323, respectively. A single nucleotide (at position 213) separates the direct repeats in MoMSV LTR. In contrast, the longer direct

repeat associated with MCF 2 proviral DNA begins 141 bp from the 5′ terminus of the LTR (at position 153) and is separated from the repeating unit by a 4-bp intervening sequence (Fig. 8A). In addition to the difference in the size of the direct repeat between MCF 2 and MoMSV proviral DNAs, extensive nucleotide heterogeneity (approximately 33%) was observed in the U_3 region of the LTR, whereas only about 13% nucleotide dissimilarity occurred in the U_5 region. The variation in the U_3 region has also been demonstrated for several avian retroviral DNAs (18, 41). Regardless of the heterogeneity in the LTRs, certain sequences located in either the U_3 or U_5 region were highly conserved. These include the 11-bp inverted terminal repeats, the polyadenylation signal sequence at positions 565 to 570 located 15 nucleotides upstream from the dinucleotide C-A at position 586, which represents the polyadenylation site (30), and the putative promoter-like sequence (44) at positions 489 to 496.

Variations in the size and organization of the LTRs found in MuLV proviral DNAs have been previously described (32, 33). It has been suggested that two size classes of LTRs are present in ecotropic proviral DNAs which differ from one another by the length of the direct repeat which resembles either MoMSV (73 bp) or AKR ecotropic MuLV (101 bp) DNAs (32). A curious but relevant feature of the analysis of Rands et al. (32) of ecotropic proviruses was the finding that two proviral DNAs contained LTRs of both size classes. This result is similar to that obtained for MCF 1 and MCF 2 proviruses which were molecularly cloned from cells infected with AKR MCF 247 MuLV, cloned by endpoint dilution. Our nucleotide sequencing results clearly show, however, that the difference in LTR size is due to the occurrence of the direct repeat in MCF 2 DNA. Our results do not address the question regarding the presence or absence of the direct repeat in the LTR of the infectious MCF 247 provirus. In this regard, a functional LTR without a direct repeat has been identified in a clone of MoMuLV DNA (2). However, it is tempting to speculate that since the 6.8-kb *Eco*RI MCF 1 DNA segment is indistinguishable by several criteria from an identical-sized fragment present in the MCF 247 provirus and does not contain a direct repeat, the LTR of the parental MCF 247 proviral DNA may also not contain a direct repeat. It should be noted that nucleotide sequence analysis of the LTR present in a "complete" but noninfectious clone of MCF 247 proviral DNA also contains only one of the reiterated components of the direct repeat (M. Kelly, M. Lung, C. Holland, S. Chattopadhyay, D. Lowy, and N. Hopkins, personal communication).

ACKNOWLEDGMENTS

We are grateful to Janet W. Hartley for providing the MCF 247-infected mink cells. We also thank Teri L. Cecil for typing the manuscript and J. Harry Belcher for preparing the radiolabeled probes.

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